Myofilin, a protein in the thick filaments of insect muscle

Feng Qiu*, Sigrun Brendel, Paulo M. F. Cunha, Nagore Astola, Bauzhen Song, Eileen E. M. Furlong, Kevin R. Leonard and Belinda Bullard‡

European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany
*Present address: CABM, UMDNJ-Robert Wood Johnson Medical School, 679 Hoes Lane, Piscataway, NJ 08854, USA
‡Author for correspondence (e-mail: bullard@embl.de)

Accepted 19 January 2005
Journal of Cell Science 118, 1527-1536 Published by The Company of Biologists 2005
doi:10.1242/jcs.02281

Summary

Thick filaments in striated muscle are myosin polymers with a length and diameter that depend on the fibre type. In invertebrates, the length of the thick filaments varies widely in different muscles and additional proteins control filament assembly. Thick filaments in asynchronous insect flight muscle have an extremely regular structure, which is likely to be essential for the oscillatory contraction of these muscles. The factors controlling the assembly of thick filaments in insect flight muscle are not known. We previously identified a thick filament core protein, zeelin 1, in *Lethocerus* flight and non-flight muscles. This has been sequenced, and the corresponding proteins in *Drosophila* and *Anopheles* have been identified. The protein has been re-named myofilin. Zeelin 2, which is on the outside of *Lethocerus* flight muscle thick filaments, has been sequenced and because of the similarity to *Drosophila* flightin, is re-named flightin. In *Drosophila* flight muscle, myofilin has a molecular weight of 20 kDa and is one of five isoforms produced from a single gene. In situ hybridisation of *Drosophila* embryos showed that myofilin RNA is first expressed late in embryogenesis at stage 15, a little later than myosin. Antibody to myofilin labelled the entire A-band, except for the H-zone, in cryosections of flight and non-flight muscle. The periodicity of myofilin in *Drosophila* flight muscle thick filaments was found to be 30 nm by measuring the spacing of gold particles in labelled cryosections; this is about twice the 14.5 nm spacing of myosin molecules. The molar ratio of myofilin to myosin in indirect flight muscle is 1:2, which is the same as that of flightin. We propose a model for the association of these proteins in thick filaments, which is consistent with the periodicity and stoichiometry. Myofilin is probably needed for filament assembly in all muscles, and flightin for stability of flight muscle thick filaments in adult flies.

Key words: Thick filament, Myosin, *Drosophila*, *Lethocerus*, Core protein, Insect flight muscle

Introduction

The precise assembly of myosin molecules into the thick filaments of striated muscle is characteristic of particular muscle types, and is important for their function. In striated muscle, the rod parts of the molecules form the backbone of the filament and the heads project from the filament to form crossbridges that interact with actin in the thin filament. Thick filaments in striated muscles of the same fibre type are uniform in length and diameter. In vertebrate striated muscle, titin is associated with the filaments and may act as a template for thick filament assembly, ensuring all filaments are the same (Fürst et al., 1988; Trinick, 1996; Gregorio et al., 1999). The sarcomeres of invertebrate muscles vary widely in length from one muscle type to another and there is no titin-like protein long enough to act as a template for thick filaments, which can be up to 10 μm long. The assembly of invertebrate thick filaments is controlled by additional proteins, either within the core of the filaments, or arranged periodically on the outside. Paramyosin is commonly found in the core (Szent-Györgyi et al., 1971; Epstein et al., 1985). Paramyosin is a coiled-coil helical protein, similar to the rod region of myosin, and the anti-parallel arrangement of paramyosin molecules in the middle of the sarcomere is thought to nucleate formation of the bipolar thick filament (Cohen and Holmes, 1963; Szent-Györgyi et al., 1971). Several proteins have been identified in the core of thick filaments in the muscles of the nematode, *Caenorhabditis elegans* (Epstein et al., 1995; Liu et al., 1998). In a model proposed by Epstein and colleagues, the core of thick filaments in the body wall muscle consists of sub-filaments of paramyosin, which surround, and are associated with, three central core proteins, α-, β- and γ-filagenin (Barral and Epstein, 1999). These core proteins are essential for the correct assembly of myosin molecules to form thick filaments of the appropriate length at different developmental stages.

The indirect flight muscle (IFM) of insects has an unusually regular hexagonal lattice of thick and thin filaments. Thick filaments are in exact lateral register across the sarcomere, and crossbridges arising from the thick filament match actin target sites on the thin filament (Wray, 1979a; AL-Khayat et al., 2003). Oscillatory contraction in IFM is likely to depend on the precise geometry of thick filaments, and the assembly and maintenance of the structure would need accessory proteins. Two insect species are frequently used in studies of IFM: *Lethocerus* (waterbug) for investigation of muscle structure

Journal of Cell Science

Journal of Cell Science

and biochemistry, and *Drosophila* for developmental studies and genetics. We have studied thick filament proteins in both insects.

Little is known about the proteins in the core of insect thick filaments. Paramyosin occurs in varying amounts in the filament cores of different insect muscles, and there is in addition, a shorter form of paramyosin (mini-paramyosin) (Becker et al., 1992; Maroto et al., 1995). Mini-paramyosin is in both flight and non-flight muscles of *Drosophila* and *Lethocerus*; in *Drosophila* IFM, the protein is concentrated at the ends of the thick filaments and in the H-zone, whereas it is uniformly distributed in the filaments of other muscles (Maroto et al., 1996). Sub-filaments have been observed within the core of thick filaments in electron micrograph images of transverse sections of IFM, and these are thought to contain paramyosin (Beinbrech et al., 1992). No protein with sequence homologous to the nematode filagenins has been identified in the *Drosophila* genome. We previously described two proteins associated with the thick filaments of *Lethocerus* muscle (Ferguson et al., 1994). Zeelin 1 is present in both flight and leg muscle, and zeelin 2 is only in flight muscle. Zeelin 1 is inside the thick filament and zeelin 2 is on the outside. This was shown in two ways. First, in isolated IFM myofibrils, zeelin1 was not fully exposed to antibody label unless myosin was first extracted from thick filaments, whereas, zeelin 2 was clearly labelled in myofibrils with intact thick filaments. Second, analysis of electron micrograph images of oblique sections of IFM labelled with antibody and Protein A-gold, showed that zeelin 1 was restricted to the 'split myosin' part of the section, which cuts through the thick filament core; the same pattern of labelling was seen with anti-paramyosin. Zeelin 2 had a broader distribution where the section cut through the surface of the thick filament.

The proteins were called zeelins because they were originally isolated with Z-discs (Bullard and Sainsbury, 1977; Sainsbury and Bullard, 1980). Subsequently, we showed that in glycerinated muscle, zeelins migrate from the thick filament to the Z-disc (Ferguson et al., 1994). We show here that the sequence of *Lethocerus* zeelin 2 is similar to that of flightin in *Drosophila* flight muscle. Flightin has been characterised by Vigoreaux and colleagues (Vigoreaux et al., 1993; Vigoreaux and Perry, 1994; Vigoreaux et al., 1998; Reedy et al., 2000; Henkin et al., 2004). It is associated exclusively with flight muscle thick filaments and is essential for the correct assembly and the stability of these filaments. We propose to call *Lethocerus* zeelin 2, flightin, and zeelin1 will be called myofilin, as it is a protein of the myosin-containing thick filament.

The aim of this investigation was to identify proteins that might be responsible for the regular assembly of insect thick filaments, particularly those in IFM. In order to understand the architecture and function of the thick filament, it is necessary to know what proteins are there and how they interact with each other. Previous work on the proteins in *Lethocerus* IFM thick filaments has been extended to *Drosophila*. The development of *Drosophila* muscle can be studied by molecular genetics and the function of particular proteins investigated in mutants. Myofilin is a potential target for the generation of mutants.

We have used several different approaches to the problem of thick filament organisation. We show that myofilin is found in *Lethocerus* and *Drosophila* thick filaments of all muscle types examined. The timing of expression of myofilin during embryogenesis and the muscle types in which it is expressed have been determined by in situ hybridisation of *Drosophila* embryos. Immunolabelling *Drosophila* flight muscle showed a periodicity in myofilin distribution on the thick filament. Based on this, and the stoichiometry, we propose a model in which myofilin is associated with myosin in the core of the thick filament, and flightin is on the surface.

Materials and Methods

Fly stocks

Wild-type *Drosophila melanogaster* were *Oregon-R* strain. A mutant lacking IFM myosin and thick filaments was *Mhc*^{7} *(Ifm(2)2)*. Both fly stocks were obtained from J. C. Sparrow (University of York, UK).

Isolation of myofilin and flightin from *Lethocerus* flight muscle

Lethocerus indicus were collected in Thailand by T. Poulsen. Myofilin (zeelin 1) and flightin (zeelin 2) were isolated from the indirect flight muscle as described previously (Ferguson et al., 1994). Proteins insoluble at high ionic strength were dissolved in 8 M urea and the two proteins were separated by gel filtration on S-Sepharose.

Molecular cloning and sequencing

Peptide sequences of *Lethocerus* myofilin and flightin from IFM were obtained by tandem mass spectrometry, using a Q-Tof mass spectrophotometer (Micromass). Proteins were alkylated and digested overnight with trypsin; peptide sequences obtained by mass spectrometry were compared with those in a comprehensive protein database using the program PeptideSearch.

For RACE reactions, mRNA was prepared from 100 μg total RNA isolated from *Lethocerus* IFM, using an Oligotex^{TM} purification kit (Qiagen). Double-stranded cDNA was prepared using a Marathon cDNA amplification kit from Clontech, according to the manufacturer's instructions. *D. melanogaster* cDNA was prepared from adult mRNA obtained from Clontech.

Myofilin

In order to clone the full-length *Lethocerus* IFM myofilin, seven sets of degenerate primers were designed from seven different peptide sequences obtained by mass spectrometry. Degenerate PCR reactions with *Lethocerus* cDNA prepared from IFM were performed with 49 combinations of the primers. All PCR products were sequenced, and the peptide sequences were aligned. One PCR fragment (368 bp) was obtained using two degenerate primers corresponding to peptide sequences ICSSIYDDPLHAA (upstream) and HPEADFAASR (downstream). The full-length myofilin cDNA was cloned by 5′-RACE and 3′-RACE amplification. The 5′-RACE primer was GTAAGGTAACCGGGCGTAATAAC and the 3′-RACE primer was AAACACGATGAACATAGCTACTTCG, based on the sequence of the 368 bp PCR fragment (Qiu et al., 2003). The EMBL database accession number for *Lethocerus* myofilin (zeelin 1) is AJ535264.

Drosophila sequences homologous to *Lethocerus* myofilin (protein sequence) were found by searching the *Drosophila* EST database (www.fruitfly.org). Three alternatively spliced isoforms, AY058374, AY071198 and BT001435 were found in the EST database. Another two alternatively spliced isoforms were amplified from cDNA prepared from whole *Drosophila* and cloned using PCR primers: 5′-ATGTTCAAAAACCACTTGGAAATG-3′ (upstream) and 5′-TTA-CAAAATTGAGCGGTGAGG-3′ (downstream), based on AY058374 cDNA sequence. The exon-intron structure of the *Drosophila* myofilin gene was determined by aligning the cDNA sequences with the

Journal of Cell Science

genomic sequence in the database. The EMBL database accession number for *Drosophila* myofilin is AJ535260.

Flightin

Four peptide sequences of *Lethocerus* flightin were obtained by mass spectrometry. Two sequences were chosen that showed the highest similarity to sequence in *Drosophila* flightin. The peptide sequences VRPPPAPK and YYDDYLDF were used to design upstream and downstream degenerate primers, and a 135 bp PCR fragment was amplified from *Lethocerus* IFM cDNA. Sequence of the full-length cDNA for flightin in IFM was obtained using 5′-RACE primer TCGTACTGCAGGAACGTCG and 3′-RACE primer CCTGG-AGCCGATCAAGCTAGTC, based on the sequence of the 135 bp PCR fragment. Sequences were aligned using the program ClustalX. The EMBL database accession number for *Lethocerus* flightin (zeelin 2) is AJ877174.

Expression of recombinant protein and preparation of antibodies

The entire *Drosophila* myofilin coding region amplified from the EST clone AY058374 was inserted into the *Nco*I/*Eco*RI site of a modified pET24d (M11) expression vector (Novagen), containing an N-terminal (His)6 tag followed by a TEV protease cleavage site. Recombinant protein was grown in *E. coli* BL21(DE3) cells (Stratagene). Myofilin was in inclusion bodies and was purified from the insoluble pellet of lysed cells on a Ni-NTA agarose column (Qiagen) in 8 M urea, 50 mM K-phosphate buffer, pH 8. Myofilin was eluted from the column by the addition of 200 mM imidazole to the 8 M urea buffer. Myofilin is insoluble in non-denaturing solutions and polyclonal antibody was raised by immunising rabbits with a suspension of myofilin in 2 mM Tris-HCl (pH 8.0), 2 mM DTT. Antiserum raised against *Drosophila* flightin was a gift from J. Vigoreaux (Reedy et al., 2000). Monoclonal antibody to the S2 region of *Lethocerus* myosin (MAC 147) was raised in rats and is subclass IgG2a (A. Hutchings, B.B. and G. W. Butcher, unpublished).

Immunocytochemistry

Embryos were fixed for 20 minutes in heptane and 4% formaldehyde in phosphate-buffered saline, pH 7.0 (PBS), and devitellinised in a 1:1 mixture of heptane and methanol with vigorous shaking. Rabbit antiserum raised against *Drosophila* myofilin was pre-absorbed against fixed wild-type embryos. Embryos were incubated with a 1:100 dilution of antibody at 4°C overnight. Embryos were then washed with PBT-BSA (0.1% Triton X-100, 0.2% BSA in PBS) and blocked with PBT-BSA with 0.2% normal goat serum. Biotinylated anti-rabbit secondary antibody was used at a dilution of 1:400 in combination with Vector Elite ABC kit (Vector Laboratories, CA) and developed with DAB substrate (Vector Laboratories, CA).

In situ hybridisation

An antisense RNA probe was made from the full-length *Drosophila* EST clone AY058374. 1 μg *Hin*dIII linearised DNA was incubated at 37°C for 2 hours with 2 μl Dig RNA labelling mix (Roche), 2 μl 10× transcription buffer, 1 μl T7 RNA polymerase, 1 μl RNase inhibitor in a final volume of 20 μl with DEPC-treated water. The probe was fragmented by incubating with 2× carbonation buffer (120 mM Na_2CO_3, 80 mM $NaHCO_3$, pH 10.2) at 65°C for 15 minutes. The precipitated probe was resuspended in 100 μl hybridisation buffer (50% formamide, 5× SSC, pH 5.0, 100 μg/ml salmon sperm, 50 μg/ml heparin, 0.1% Tween 20) and stored at –20°C. Embryos were incubated with digoxigenin-labelled RNA probes at 1:50 dilution in hybridisation buffer at 65°C overnight. Following repeated washing at 65°C with 50% formamide, 5× SSC, the embryos were rehydrated in step-wise fashion and incubated in blocking solution (PBS, 0.1% Tween-20, 0.2% BSA). The embryos were then incubated at 4°C overnight in 250 μl blocking solution with a 1:2000 final concentration of pre-adsorbed anti-digoxigenin-alkaline phosphatase antibody (Roche). Following washes in blocking solution, the embryos were developed with NBT and X-Phosphate (Roche) in staining solution (0.1 M NaCl, 0.1 M Tris-HCl, pH 9.0, 0.05 M $MgCl_2$, 0.1% Tween 20).

Northern blotting

RNA was extracted from *Drosophila* at different developmental stages using Trizol (Invitrogen). 30 μg total RNA for each sample was loaded on to a 1% agarose gel containing formaldehyde. Northern blots were processed following standard molecular biology procedures. Probes were synthesised using the RadPrime DNA Labeling System (Invitrogen) purified with ProbeQuant™ G-50 Micro Columns (Amersham), according to the manufacturer's recommendations. Probes used in the northern blots, with the corresponding genes in parentheses, were: GH14252 (*myofilin*) and RH03940 (*Rp49*). *Rp49* (also called *RpL32* and CG7939) was used as a loading control. GH14252 corresponds to EST clone AY058374 (DmMf3 in Fig. 2B). DNA sequence was verified at the EMBL Genomics Core Facility.

Electrophoresis and immunoblotting

Drosophila thoraces were removed from wild-type and mutant flies and immediately put into liquid nitrogen. Thoraces were homogenised in an Eppendorf tube while frozen and heated to 95°C in Laemmli sample buffer with 1 μM leupeptin. Dorsal-longitudinal and dorso-ventral indirect flight muscles were dissected from half thoraces derived from about 30 wild-type or mutant *Drosophila* in rigor solution (0.1 M NaCl, 20 mM sodium phosphate, pH 6.8, 5 mM $MgCl_2$, 5 mM EGTA, 5 mM NaN_3) with 0.5% Triton X-100 and 1 μM leupeptin. Samples were run on 5 cm SDS-PAGE gels with 15% acrylamide. Proteins were transferred to nitrocellulose with a semi-dry blotting apparatus (ATTA, Genetic Research Instrumentation) for 1 hour at 900 mA. Blots were incubated in antiserum raised against myofilin (diluted 1:1000), followed by goat anti-rabbit secondary antibody (Sigma) (diluted 1:50,000), and developed with a chemiluminescent substrate (ECL, Amersham).

Myosin binding to myofilin was detected by a blot overlay assay. *Lethocerus* IFM myofibrils were washed in rigor solution with 0.5% Triton X-100. Samples were run on 10% SDS-PAGE gels and transferred to nitrocellulose as above. Blots were incubated in blocking buffer (PBS, 0.2% Triton-X100, 5% non-fat milk) or blocking buffer with *Lethocerus* myosin (0.1 mg/ml). After washing, blots were incubated in hybridoma supernatant of monoclonal antibody MAC 147 (diluted 1:10), and then in goat anti-rat secondary antibody (Sigma) (diluted 1: 50,000). Blots were developed as above.

Immunoelectron microscopy

Thoraces were dissected from adult *Drosophila* and divided into two by a vertical cut along the anterior-posterior axis. Half thoraces were fixed in 4% paraformaldehyde in relaxing solution (rigor solution with 5 mm ATP) for 30 minutes on ice and then infused with 2.1 M sucrose in rigor buffer at room temperature for 30 minutes. The thoraces were placed on copper stubs with the cuticle in contact with the stub and the IFM and other thoracic muscles facing upwards. Stubs were plunged into liquid N_2. Cryosections were labelled with antiserum (diluted 1:10) raised against myofilin, followed by Protein A-gold (10 nm) (Lakey et al., 1990). Images were taken with a Technai Biotwin microscope at 100 kV and recorded digitally with a Gatan CCD camera. The positions of gold particles were measured using NIH Image software. Histograms were calculated using Kaleidograph

Journal of Cell Science

(Albeck Software). The distribution of gold particle separations was estimated as described previously (Newman et al., 1992).

Results

Sequence of *Lethocerus* and *Drosophila* myofilin

The first step in sequencing both *Lethocerus* and *Drosophila* myofilin was to obtain the sequences of peptides derived from the protein isolated from *Lethocerus* IFM (Ferguson et al., 1994). Using primers designed from these sequences, the full-length *Lethocerus* IFM myofilin was cloned by PCR and RACE reactions with *Lethocerus* IFM cDNA. The sequences of homologous isoforms in *Drosophila* were identified in the EST database and were also obtained by PCR reactions with cDNA derived from whole flies. No vertebrate proteins with homology to myofilin were found in the database.

Five myofilin transcripts are produced from the *Drosophila* gene by alternative splicing. The organisation of ORFs in the gene and the exon composition of transcripts are shown in Fig. 1A and B. The gene contains nine exons; the start codon is in exon 2 and there are stop codons in exons 5, 8 and 9. Exons 5, 6 and 7 have internal splice sites and isoforms contain long or short versions of these exons. The *Drosophila* myofilin gene maps to chromosome position 3R 88E2, which is close to several genes expressed specifically in IFM (Karlik et al., 1984; Fyrberg, 1985; Bernstein et al., 1993). This suggests that there may be some coordination in the regulation of the expression of these genes (Spellman and Rubin, 2002). No mutants have been identified at this chromosome position. The FlyBase gene number is CG6803.

The sequence of one isoform of *Lethocerus* myofilin was obtained using cDNA from IFM. There are multiple isoforms, as in *Drosophila*; however we did not attempt to clone isoforms from cDNA derived from other *Lethocerus* muscle types. The predicted molecular mass of the *Lethocerus* myofilin is 30.3 kDa, which is lower than the mass of 35 kDa estimated from SDS-PAGE (Ferguson et al., 1994); the slow migration on SDS-PAGE is probably due to the relatively high proportion of proline in the sequence (10% of residues). The isoform in *Drosophila* IFM is DmMf2, which is about 20 kDa (see below).

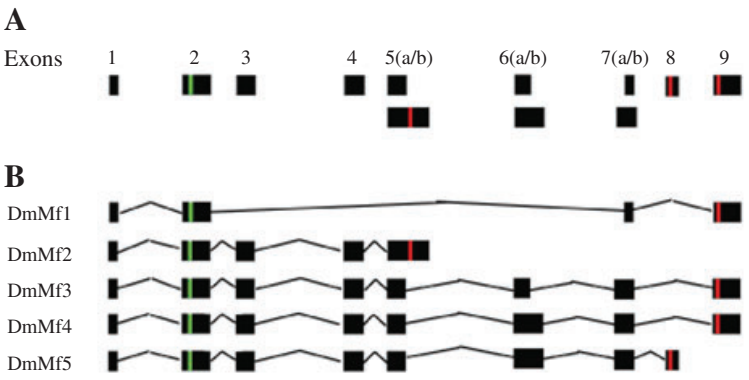

Fig. 1. Alternative splicing of the *D. melanogaster* myofilin gene. (A) The top line shows the exon-intron organisation of the myofilin gene, which contains nine exons. The start codon (green) is found in exon 2, stop codons (red) are found in exons 5, 8 and 9. Exons 5, 6, and 7 have internal splice sites, alternative versions are indicated by a (upper) and b (lower). (B) Schematic representation of the structure of mRNAs encoded by the myofilin gene. The exons present in the different isoforms DmMf1 to DmMf5 are shown.

The EST database for the mosquito, *Anopheles gambiae*, contains sequence with homology to *Lethocerus* and *Drosophila* myofilin. The amino acid sequences of the largest *Drosophila* isoform (DmMf5) and the IFM isoform (DmMf2) are compared with those of the *Lethocerus* IFM isoform (LiMf2) and the single *Anopheles* sequence (Fig. 2A). *Anopheles* myofilin is predicted to be 18.4 kDa and, like DmMf2 and LiMf2, it lacks the long extra sequence in the middle of the protein that is present in DmMf5; it is therefore called AgMf2. The *Lethocerus* and *Anopheles* sequences are most similar to the sequence of *Drosophila* DmMf2 in the N-terminal half of the molecule, and less so in the region C-terminal to the extra sequence in DmMf5. *Lethocerus* IFM myofilin has three regions of inserted sequence, and extra sequence at the C-terminus compared to *Drosophila* IFM myofilin (DmMf2) and *Anopheles* AgMf2. All myofilins are basic, with isoelectric points: DmMf2, 9.1; DmMf5, 10.1; AgMf2, 9.3; and LiMf2, 9.2. There are four stretches of sequence predicted to be helical in the largest *Drosophila* isoform (Fig. 2). The stretch near the N-terminus is conserved in *Lethocerus* and the diptera; further downstream there is a helical stretch that is conserved in the diptera but not *Lethocerus*. The next two helical sequences are only in the longest *Drosophila* isoform. There is very little predicted β-sheet structure and the molecule is expected to be globular rather than fibrous.

The sequence of filagenins in the core of thick filaments in *C. elegans* might be expected to have some similarity with insect myofilin. But filagenin sequences have only 16%, or less, identity with DmMf2. This is in contrast to LiMf2, which has 59% identity with DmMf2, and AgMf2, which has 67% identity. The sequence of β-filagenin is compared with insect myofilins in Fig. 2A. Single residues identical to those in *Lethocerus* or the diptera are distributed throughout the molecule and there are no common peptides.

Lethocerus and *Drosophila* flightin

The sequence of *Lethocerus* flightin obtained here was compared with that of *Drosophila* flightin (Vigoreaux et al., 1993) (Fig. 2B). *Drosophila* and *Lethocerus* flightin have regions of sequence in common, although the entire sequences are only 35% identical. Flightin is about the same size in the two insects: 19 kDa in *Lethocerus* and 20 kDa in *Drosophila*. The limited sequence similarity and the pattern of distribution on the thick filament (see below), means the proteins are likely to be homologues.

Isoforms of myofilin in *Drosophila* muscles

We have shown previously that myofilin is associated with the thick filaments of different muscle types in *Lethocerus* (Ferguson et al., 1994). We aimed to determine which of the *Drosophila* myofilin isoforms is in IFM thick filaments. Isoforms in the muscles of the *Drosophila* thorax were detected using antiserum raised against recombinant protein. The antiserum reacted strongly with three proteins of 20-42 kDa, and weakly with a protein of about 44 kDa (Fig. 3). There was a very weakly labelled protein of ~7.5 kDa, which may

Journal of Cell Science

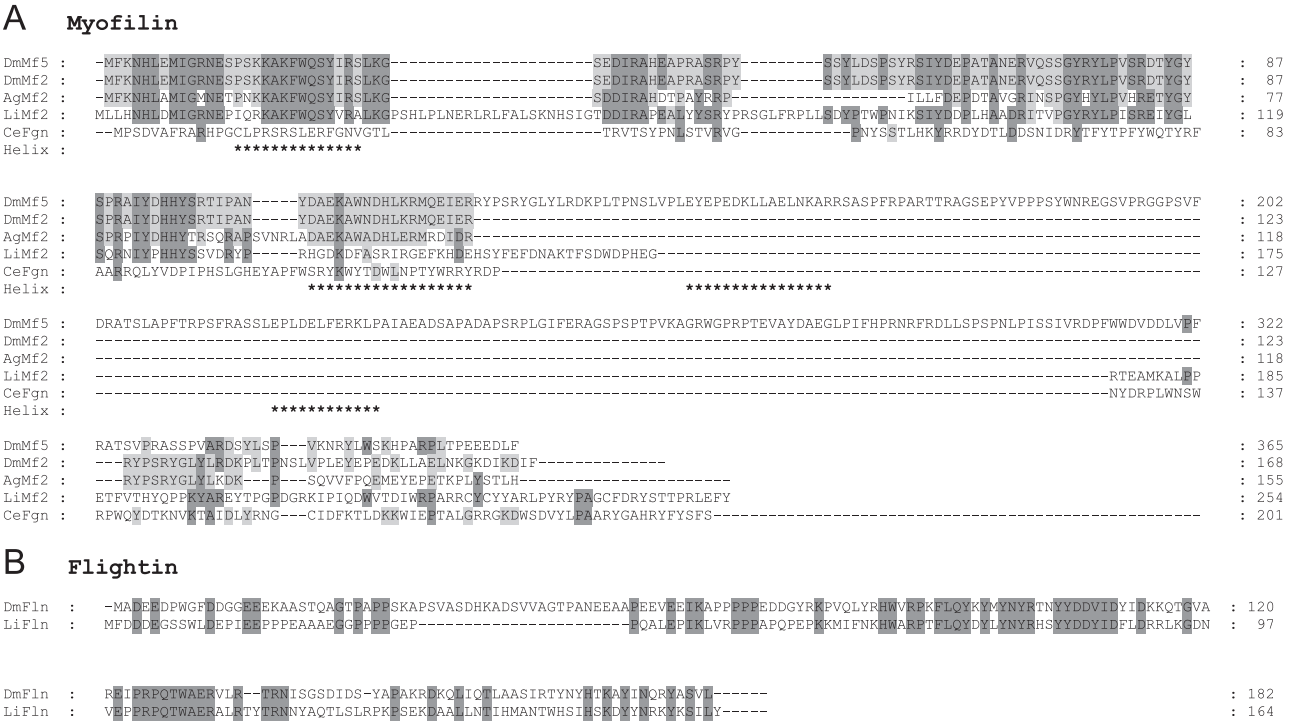

Fig. 2. Comparison of the sequences of myofilin and the sequences of flightin in different insects. (A) Alignment of myofilin amino acid sequences in *Drosophila melanogaster* (isoforms DmMf5 and DmMf2), *Anopheles gambiae* (AgMf2) and *Lethocerus indicus* (LiMf2). *Drosophila* and *Lethocerus* myofilin sequences were obtained as described in the Materials and Methods. The homologous sequence in *Anopheles* is from the EST database (BM64635). *Caenorhabditis elegans* β-filagenin sequence is shown below the myofilin sequences (Liu et al., 1998). Stars below the sequence are regions in DmMf5 predicted to be helical. (B) Alignment of flightin sequences in *D. melanogaster* (DmFln) and *L. indicus* (LiFln). No flightin sequence was found in the *Anopheles* genome. In both A and B, residues that are identical in *Lethocerus* and *Drosophila* or *Anopheles* or *C. elegans*, or all four, are shaded dark grey; other identical residues are shaded light grey. This illustrates the extent of homology between the sequences of the hemipteran, *Lethocerus*, and the two dipterans, *Drosophila* and *Anopheles*, as well as that between the sequences of individual insect species; *C. elegans* β-filagenin is included for comparison with a nematode.

be a minor isoform in the thorax. The isoform of ~20 kDa (DmMf2) was labelled most strongly in the wild-type thorax, suggesting it is in IFMs, which make up the largest muscle mass in the thorax. The specific expression of DmMf2 in IFM was shown by an immunoblot of the mutant, *Mhc*^{7}. In these flies, a mutation in IFM myosin eliminates expression in IFM and there are no thick filaments; other muscle types are normal. Thoraces of *Mhc*^{7} had all the myofilin isoforms detected in wild-type thoraces except the 20 kDa isoform (Fig. 3). IFMs isolated from wild-type flies had only

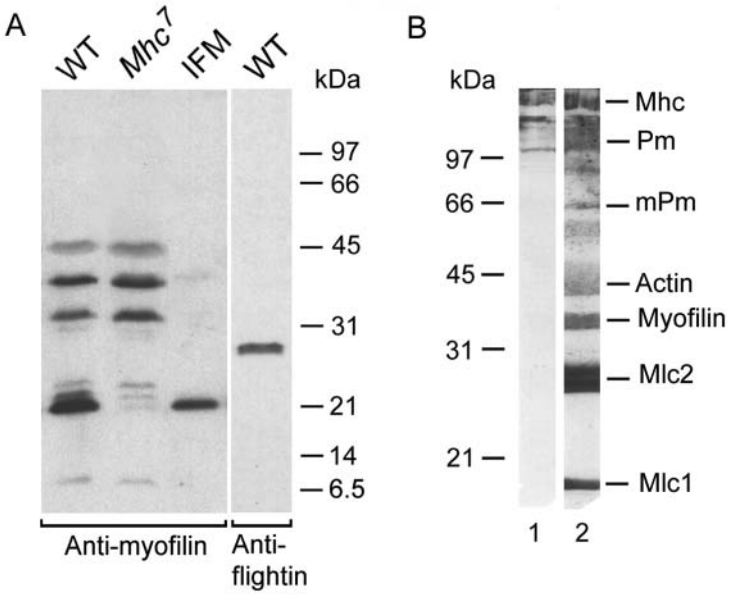

Fig. 3. Isoforms of myofilin in *Drosophila* and myosin binding to myofilin. (A) An immunoblot of thoraces from wild-type *Drosophila* (WT) and mutant flies lacking thick filaments in the IFM (*Mhc*^{7}), and isolated wild-type flight muscle (IFM) was incubated with anti-myofilin antiserum. There are five isoforms of myofilin in muscles of the thorax. *Mhc*^{7} lacks the 20 kDa isoform, which is the sole isoform in IFM. The strip on the right shows a blot of thoraces from wild-type flies incubated with anti-flightin. (B) Overlay assay of myosin binding to immunoblots of *Lethocerus* IFM. Lane1 was incubated in myosin antibody (MAC 147); lane 2 was incubated in buffer containing *Lethocerus* myosin (0.1 mg/ml) and then in myosin antibody. Myosin bound to myofilin and to other proteins (paramyosin, Pm; mini-paramyosin, mPm; and actin). Myofilin (30 kDa) has an anomalously low migration rate on SDS-PAGE gels. Myosin also bound strongly to the two myosin light chains (Mlc1 and Mlc2); there are three variants of Mlc2, which differ in the extent of phosphorylation and have slightly different mobilities. Myosin did not bind to flightin (23 kDa). Mhc, myosin heavy chain.

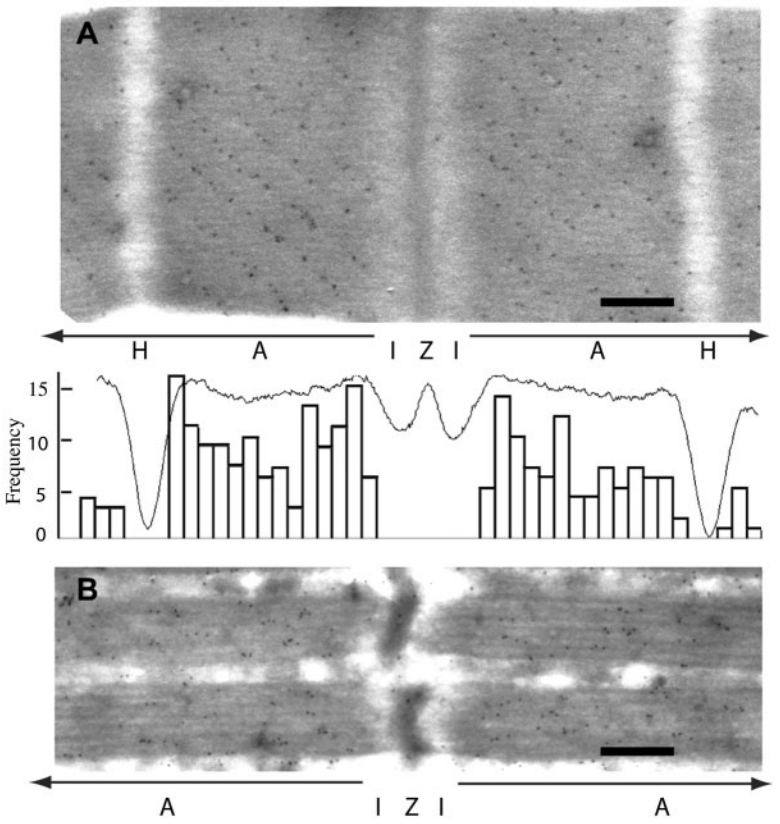

Fig. 4. The position of myofilin in *Drosophila* muscles. (A) Thin, slightly oblique cryosection of *Drosophila* IFM labelled with anti-myofilin and Protein A-gold. The fibre is slightly stretched to increase the length of the I-band. Gold particles are in diagonal lines across the sarcomere; this results from exposure of epitopes by the knife cutting through the regular thick filament lattice (Ferguson et al., 1994). The positions of H-zone (H), A-band (A), I-band (I), Z-band (Z) are shown. Horizontal lines mark the extent of the A-band. Beneath the micrograph is a histogram showing the distribution of gold particles and the corresponding density profile across the sarcomere. Gold particles extend to the edges of the A-band and H-zone. (B) Cryosection of non-flight muscle labelled with anti-myofilin and Protein A-gold. In this case the myofibrils are narrower and the sarcomeres longer, so that the H-zones are not visible in this micrograph. Labelling is less regular, but again extends to the end of the A-band. Bar, 200 nm.

the 20 kDa isoform. These results show that there are myofilin isoforms of varying size in thoracic muscles of *Drosophila* and that DmMf2 is the sole isoform in the IFM.

Drosophila flightin, like DmMf2, is associated exclusively with IFM thick filaments. Antibody to flightin labelled a protein of apparent molecular mass ~28 kDa in wild-type thoraces, which did not correspond to any myofilin isoform. Myofilin and flightin antibodies did not label the same proteins, showing they were specific to their target antigens (Fig. 3). Therefore myofilin and flightin are two distinct proteins associated with the IFM thick filament.

Binding of myosin to myofilin

Both myofilin and flightin are insoluble in non-denaturing solvents, so it is not possible to measure binding properties of the proteins in solution. We showed previously that, in solutions of low ionic strength, *Lethocerus* myofilin self-associates to form micelles and flightin forms filaments; the two proteins do not co-polymerise (Ferguson et al., 1994). Binding between myosin and myofilin and flightin was investigated in a blot overlay assay. *Lethocerus* IFM myofibrils were run on an SDS-PAGE gel and a blot of the gel was incubated in myosin. In this assay, myosin bound to myofilin but not to flightin (Fig. 3B). Myosin bound to several other proteins, including paramyosin and mini-paramyosin. The myosin light chains, Mlc1 and Mlc2, were strongly labelled. The antibody used to detect myosin binding was a monoclonal antibody specific to the S2 region of the myosin heavy chain (Mhc). Therefore, added Mhc was binding to Mlc1 and Mlc2. This suggests that either the myosin in which the blot was incubated did not have the full complement of light chains, or light chains on the blot displaced those in the added myosin. Although the blot overlay assay did not show any binding between myosin and flightin, *Drosophila* flightin has been shown to bind to the rod region of myosin in other solid-state binding assays (Ayer and Vigoreaux, 2003).

Distribution of myofilin in *Drosophila* muscles

The position of myofilin in *Drosophila* IFM was determined by labelling cryosections with antibody and Protein A-gold. Sarcomeres were slightly stretched so that the ends of the A-band could be seen clearly. Anti-myofilin labelled the A-band up to the ends of the thick filaments; there was no label in the short I-band or in the H-zone in the central region of the sarcomere (Fig. 4A). The histogram below the electron micrograph shows that the distribution of myofilin across the A-band is fairly uniform. The narrow diagonal banded appearance of the gold particles is characteristic of label on an antigen restricted to the core of the thick filaments. The section is slightly oblique and is labelled on one side only. The diagonal bands are due to intersection of the cutting plane with the regular lattice of the IFM. The pattern of labelling is similar to that seen in oblique sections of *Lethocerus* IFM labelled with anti-zeelin 1 (Ferguson et al., 1994). Gold label is confined to regions where the plane of the section cuts through the centre of the thick filament (split myosin region). A model for the pattern resulting from split myosin labelling of a core protein such as paramyosin, compared to the broader distribution of label on flightin (zeelin 2), has been described previously (Ferguson et al., 1994).

The label on sections of *Drosophila* leg muscle was less regular but also extended close to the end of the A-band (Fig. 4B). The pattern of myofilin labelling in *Drosophila* IFM differed from the labelling seen with anti-flightin. In the IFM of both *Lethocerus* (Ferguson et al., 1994) and *Drosophila* (Reedy et al., 2000), anti-flightin labels a broad region of the sarcomere either side of the H-zone. The label does not reach the end of the A-band.

Measurement of the separation of gold particles along *Drosophila* IFM thick filaments in stretches that were labelled

Journal of Cell Science

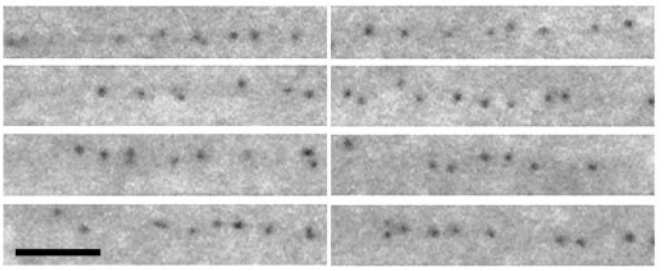

Fig. 5. Distribution of myofilin in *Drosophila* IFM thick filaments. Cryosections were labelled with anti-myofilin and Protein A-gold. Panels show selected runs of gold particles following the line of the thick filaments. The mean value for the smallest regular spacing between gold particles was at 30±5 nm (mean±s.d., *n*=42, taken from three images). Bar, 120 nm.

regularly with anti-myofilin (Fig. 5), gave a value of 30±5 nm (mean±s.d., *n*=42) for the minimum separation of myofilin molecules. *Drosophila* IFM was not labelled sufficiently regularly with anti-flightin antiserum to measure separation of flightin molecules; however, measurements of flightin spacing on *Lethocerus* IFM thick filaments, using a monoclonal antibody (Ferguson et al., 1994) gave a value of 27±8 nm (mean±s.d., *n*=24) for the spacing of flightin on IFM thick filaments. Therefore myofilin and flightin are spaced at about the same interval along the thick filament, although myofilin is on the inside of the filament and flightin is on the outside.

Expression of myofilin in the *Drosophila* embryo, larva and adult

Myofilin RNA in *Drosophila* embryos was first detected by in situ hybridisation at late stage 15, and its expression became stronger at stages 16 and 17, at the end of embryogenesis. The RNA was expressed in the somatic muscle, with little or no expression in the visceral and heart muscles (Fig. 6A,B). Myofilin protein expression was detected using the anti-myofilin antiserum. The protein is expressed in the somatic muscles of the embryo during stages 16 and 17 (Fig. 6C,D). Individual muscles were clearly seen in embryos labelled with antibody.

Northern blotting was used to detect different myofilin RNA transcripts during *Drosophila* development (Fig. 7). Equal amounts of total RNA from seven stages of embryogenesis, three stages of larval life and adult males and females were used. Myofilin RNA expression was first detected during late embryogenesis (stage 16-17), which is in agreement with the results obtained from in situ hybridisation. Expression was maintained during the three stages of larval life. In both embryo and larva there was a single transcript of ~1.3 kb, corresponding to DmMf3 (Fig. 1B). Expression of the 1.3 kb transcript continued in the adult, where there was also a second transcript of lower abundance of approximately 0.8 kb, corresponding to DmMf2. These results indicate that the DmMf3 transcript predominates during embryonic and larval development and in adults. Additional transcripts corresponding to DmMf1, DmMf4 and DmMf5 were below the level of detection.

Discussion

We have identified myofilin as a protein inside the core of thick filaments in *Lethocerus* and *Drosophila*; the protein is also found in the *Anopheles* genome and is likely be in thick filaments of mosquitoes. The existence of multiple isoforms in *Drosophila* and the presence of myofilin in embryonic muscles, as well as IFM and other muscles, suggest it is a protein with a function similar to that of the *Caenorhabditis elegans* filagenins, which control filament assembly in different muscle types (Liu et al., 1998; Liu et al., 2000). However, the sequence is unlike that of the filagenins and the protein would have different interactions with other thick filament proteins.

cDNA sequences have been obtained for *Lethocerus* IFM myofilin and five isoforms of *Drosophila* myofilin. Four of the isoforms produced by alternative splicing of nine exons are identical in the N-terminal half of the sequence and divergent in the C-terminal half. Evidently, all five isoforms are expressed in the adult fly; the apparently low amount of the smallest isoform may be real, or may be due to the absence of epitope sequences present in longer isoforms. It is likely that isoforms are expressed specifically in different muscle types in the thorax; these have varying sarcomere lengths and thick filaments of correspondingly different lengths. The sarcomeres of IFM are relatively short (3.4 μm) compared with sarcomeres of other thoracic muscles and leg muscles, some of which are more than twice as long (Leonard and Bullard, 2004). The single myofilin isoform in *Drosophila* IFM, at 20 kDa, is the

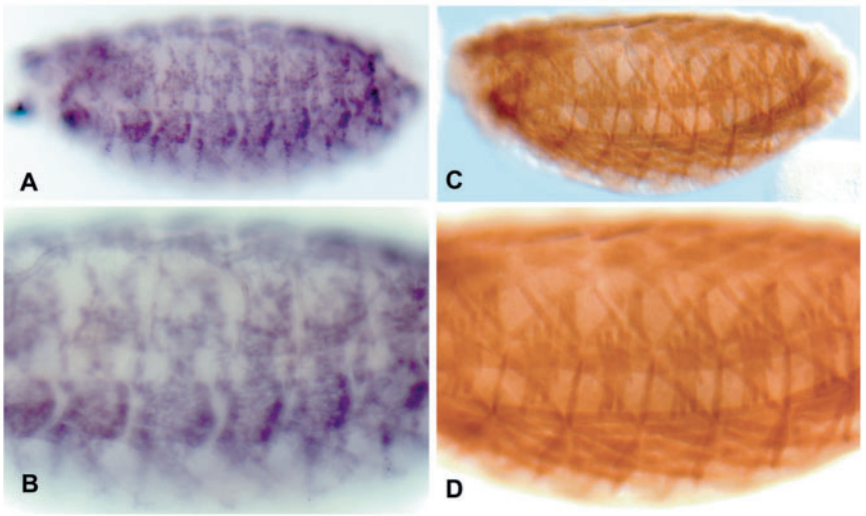

Fig. 6. Expression of *myofilin* RNA and protein in the *Drosophila* embryo. (A) Stage 15-17 embryo hybridised with a digoxigenin-labelled *myofilin* RNA probe. (B) Higher magnification of the same embryo showing labelling of somatic muscles, but not heart or visceral muscles. (C) Stage 15-17 embryo labelled with anti-myofilin antiserum, showing specific immunostaining of somatic muscles. (D) Higher magnification of the same embryo. Labelling of individual muscles can be clearly seen at this embryonic stage. Embryos are seen in lateral view with anterior to the left.

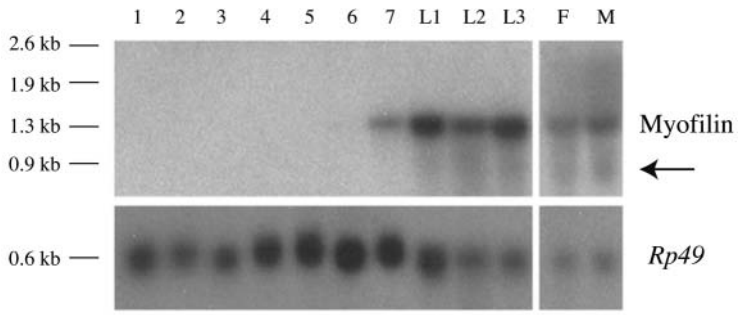

Fig. 7. Northern blot analysis of the expression of *myofilin* in *Drosophila*. Lanes 1 to 7 are stages of embryonic development as follows: lane 1, 0-1 hours (stages 1-3); lane 2, 1-3 hours (stages 1- 6); lane 3, 3-6 hours (stages 7-11); lane 4, 6-9 hours (stages 11-13); lane 5, 9-12 hours (stages 13-15); lane 6, 12-15 hours (stages 15-16); lane 7, 15-18 hours (stages 16-17). Lanes L1, L2 and L3 are first, second and third instar larvae. Lanes F and M are female and male adults. Expression of myofilin is detected as a strong band corresponding to a predicted size of 1.3 kb, starting at 15-18 hours of development or stage 16 and present in all larval stages and in the adults. A faint band is also seen that could correspond to the splice variant of predicted size 0.8 kb (arrow). This band is not detected at any stage of embryogenesis but is present throughout larval life and in the adults. *Rp49* was hybridised to the same blot as the myofilin probe (lower image) as a loading control.

smallest of the four main isoforms in the thorax. However, there is not a simple relationship between sarcomere length and the size of myofilin: the sarcomere length of *Lethocerus* IFM (2.6 μm) is shorter than that of *Drosophila* IFM and the myofilin is larger (30 kDa). The more conserved sequence in the N-terminal regions of IFM myofilins in *Lethocerus* and the two dipterans, compared to the C-terminal regions, suggests this part of the molecule has a common function in these insects.

Myofilin self-associates, forming micelles in vitro; however, this is unlikely to occur in vivo because, judging from the 30 nm periodicity on the thick filament, the molecules would be too far apart. This is shown in the model of the thick filament described below (Fig. 8). Myofilin and flightin do not bind to each other in vitro and it is unlikely they would do so in vivo, because myofilin is inside the thick filament and flightin is outside. However, both proteins bind to myosin. We have shown here that myofilin binds to the whole myosin molecule and it was shown previously that flightin binds to the middle of the light meromyosin (LMM) part of the myosin rod (Reedy et al., 2000; Ayer and Vigoreaux, 2003). The N-terminal conserved region of myofilin, which is predicted to have a relatively high helix content, may be a myosin-binding site.

Expression of myofilin and flightin during development of *Drosophila*

Although myofilin and flightin are both associated with thick filaments, they are expressed at different times during development. Myofilin RNA is detected at late embryonic stage 15 and is expressed strongly in somatic muscles at the end of embryogenesis (stage 16 and 17); the protein is detected at stage 16. Myofilin transcripts appear later than myosin heavy chain (*Mhc*) transcripts, which are detected in somatic and visceral muscles at late stage 12, and strongly expressed at stages 13 and 14 (Zhang and Bernstein, 2001). *Mhc* transcripts are detected later in cardiac muscles, at stage 15. The expression pattern of myofilin in somatic muscle is similar to that of Mhc, but myofilin was not detected in visceral and heart muscle. Although *Mhc* transcripts are detected during mid-embryogenesis (stage 13), the somatic muscle is not differentiated at this time. The process of myoblast fusion is completed by stage 15 within the somatic muscle. At this stage, the multinucleated myotubes differentiate into contractile myofibrils. Both Mhc and myofilin are highly expressed during this stage, which coincides with sarcomere assembly.

Northern blots confirmed the appearance of myofilin RNA at stage 16, late in embryogenesis. One transcript predominates in embryonic, larval and adult life. Five protein isoforms were detected in the thorax of the adult fly (Fig. 3A). As total RNA from the entire adult was used for the Northern blot analysis, it is likely that thorax-specific transcripts were below the level of detection. Alternatively, the apparent lack of RNA corresponding to all protein isoforms may be due to low levels of protein synthesis in the muscles of the adult thorax, or one or more of the protein isoforms may have different molecular masses due to post-translational modification.

Flightin is not expressed until late pupal and adult stages. RNA and protein are detected at pupal stage 8 and continue to accumulate until the fly emerges (Vigoreaux et al., 1993). The early expression of myofilin and the late expression of flightin are consistent with the positions of the two proteins in the thick filament and the specific expression of flightin in IFM. Myofilin is in different muscle types, and is likely to be in the core of thick filaments in larval as well as adult muscles. Although myofilin appears in the embryo after myosin, the correct assembly of myosin into thick filaments may need both proteins. IFM develops during the pupal stage, and flightin bound on the surface of thick filaments would be expected to appear late in metamorphosis. We did not determine the pupal stage at which myofilin appears, but it is likely to be before the appearance of flightin.

A model for the distribution of myofilin and flightin in the IFM thick filament

If myofilin (or flightin) were bound regularly to every myosin in the IFM thick filament, we would expect the spacing along the filament to correspond to the myosin head repeat (14.5 nm) and the stoichiometry to be 1:1. However, measurements made here on *Drosophila* IFM thick filaments, suggest the separation of myofilin molecules along the filament backbone is about 30 nm. The molar ratio of myofilin to myosin in IFM was estimated to be 1:2 (Ferguson et al., 1994).

Beinbrech and colleagues (Beinbrech et al., 1988) proposed a model for the IFM thick filament (based on electron micrograph images of cross-sections) in which twelve sub-filaments, each containing myosin molecules in two radial positions (called ‘inner’ and ‘outer’ here), are arranged around the periphery of the filament. Although the heads of the two types of myosin molecule are in different positions, they would still be able to make contact with actin. This model provides a

Journal of Cell Science

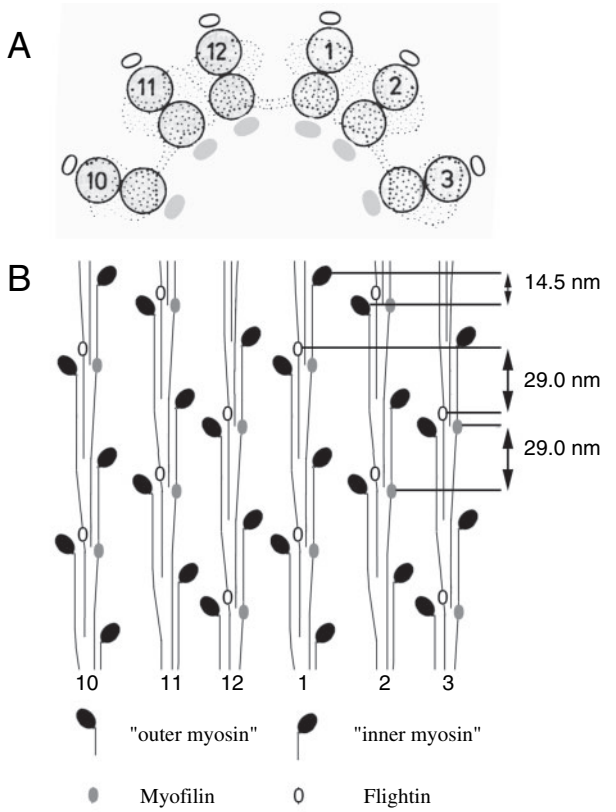

Fig. 8. A model for the arrangement of myofilin and flightin in the IFM thick filament. (A) Cross-sectional top view of half a thick filament showing the myosin sub-filaments (adapted from Beinbrech et al., 1988). In the full thick filament there would be 12 sub-filaments, here we show only numbers 1-3 and 10-12. Myofilin molecules are shown in grey on the inside of the thick filament. Flightin molecules are open symbols on the outside of the filament. (B) A side view of the thick filament lattice, again based on Beinbrech's model. Each sub-filament has myosin molecules with alternating 'outer' heads (black ellipses pointing to the left) and 'inner' heads pointing to the right. The myofilin molecules only bind to the rod region of the 'inner' myosin molecules, with the result that they are arranged at an interval of 29 nm, as shown; this is twice the 14.5 nm spacing of the myosin heads in the filament. Myofilin is placed at an arbitrary position on the myosin rod. Flightin molecules are bound to 'outer' myosin molecules about two-thirds of the way along the rod. In order to simplify the representation of the model, only one head of myosin dimers is shown, and the sub-filaments are drawn parallel to the axis of the thick filament, although evidence from X-ray diffraction suggests that they are inclined at a shallow angle to the filament (Wray, 1979b).

mechanism by which only half the potential binding sites on myosin, those on 'inner' myosin molecules, are accessible to myofilin. If half the sites are occupied, the stoichiometry of myofilin to myosin would be 1:2 and the spacing along the thick filament would be approximately 30 nm.

Fig. 8A and B shows a simplified model based on Beinbrech's model (Beinbrech et al., 1988). In Fig. 8B, each sub-filament has 'outer' myosin molecules (heads pointing to the left), which do not bind myofilin and 'inner' myosin molecules (heads pointing to the right), which bind myofilin. The resulting spacing of myofilin along the thick filament is 29 nm, or twice the myosin head repeat. The exact position of myofilin binding is not known; we have placed it here towards the myosin head. We found that flightin spacing in *Lethocerus* IFM thick filaments is about the same as that of myofilin in *Drosophila* thick filaments. In Fig. 8B, flightin is placed on the 'outer' myosin molecules; this position differs from that in a model proposed previously where flightin is on both 'inner' and 'outer' myosins (Reedy et al., 2000). The present model is in better agreement with the stoichiometry and spacing along the filament. Estimates for the molar ratio of flightin to myosin in IFM are 1:2.5 for *Lethocerus* (Ferguson et al., 1994) and between 1:1 and 1:2 for *Drosophila* (Ayer and Vigoreaux, 2003). Here we have assumed a value of 1:2.

Function of myofilin and flightin

Myofilin may be needed for correct assembly of thick filaments in all insect striated muscles. The isoform specific to IFM would determine the precise geometry of the thick filaments in these muscles. Filagenins in *C. elegans* body wall muscle control filament assembly and, like filagenins, myofilin is a basic protein in the core region of thick filaments. The relationship between the spacing of myofilin and myosin in the IFM thick filament, suggests that myofilin is associated with myosin subfilaments.

Flightin regulates thick filament and sarcomere length in IFM at a late stage of pupal development, when thick filaments are already partly assembled. Sarcomeres are abnormally long in the flightin-null mutant (fln^0) and it has been suggested that flightin is needed to terminate thick filament assembly; this may be achieved by progressive phosphorylation of flightin (Reedy et al., 2000). Flightin is essential for maintaining the intact structure of IFM thick filaments after the fly emerges from the pupa. IFMs in fln^0 are progressively degraded after eclosion (when the muscles first start to contract) and myosin is digested (Reedy et al., 2000). Both flightin and myofilin are particularly susceptible to cleavage by calpain (Bullard et al., 1990) and digestion of these proteins may be the first step in autolysis of wild-type IFM. Flightin on the outside of the IFM thick filament may keep the structure intact when sarcomeres are subjected to high frequency forces during oscillatory contraction (Henkin et al., 2004). Recent mechanical measurements on the flightin-null mutant, before degradation sets in, have shown that flightin contributes to the high resting stiffness of IFM, and is essential for the performance of oscillatory work (Henkin et al., 2004).

In conclusion, we suggest that myofilin in the core of the thick filament influences thick filament assembly, and flightin on the outside of the IFM thick filament stabilises the filament after it is assembled. The function of flightin is in general agreement with previous proposals (Reedy et al., 2000; Henkin et al., 2004), but the distribution on the thick filament differs. When the binding site of myofilin on myosin has been determined, it will be possible to produce a more precise model for association of both accessory proteins with the IFM thick filament.

We are grateful to Anne Lakey and Helen Smith for obtaining the initial clones of *Lethocerus* myofilin, and to A. Houthaeve and T. Franz (EMBL proteomics core facility) for mass spectroscopy measurements. We also thank J. Vigoreaux for anti-flightin antiserum and T. Poulsen for supplying *Lethocerus* from Thailand.

Journal of Cell Science

References

AL-Khayat, H. A., Hudson, L., Reedy, M. K., Irving, T. C. and Squire, J. M. (2003). Myosin head configuration in relaxed insect flight muscle: x-ray modeled resting cross-bridges in a pre-powerstroke state are poised for actin binding. *Biophys. J.* **85**, 1063-1079.

Ayer, G. and Vigoreaux, J. O. (2003). Flightin is a myosin rod binding protein. *Cell Biochem. Biophys.* **38**, 41-54.

Barral, J. M. and Epstein, H. F. (1999). Protein machines and self assembly in muscle organization. *Bioessays* **21**, 813-823.

Becker, K. D., O'Donnell, P. T., Heitz, J. M., Vito, M. and Bernstein, S. I. (1992). Analysis of *Drosophila* paramyosin: identification of a novel isoform which is restricted to a subset of adult muscles. *J. Cell Biol.* **116**, 669-681.

Beinbrech, G., Ashton, F. T. and Pepe, F. A. (1988). Invertebrate myosin filament: subfilament arrangement in the wall of tubular filaments of insect flight muscles. *J. Mol. Biol*. **201**, 557-565.

Beinbrech, G., Ashton, F. T. and Pepe, F. A. (1992). The invertebrate myosin filament: subfilament arrangement of the solid filaments of insect muscles. *Biophys. J*. **61**, 1495-1512.

Bernstein, S. I., O'Donnell, P. T. and Cripps, R. M. (1993). Molecular genetic analysis of muscle development, structure, and function in *Drosophila*. *Int. Rev. Cytol.* **143**, 63-152.

Bullard, B. and Sainsbury, G. M. (1977). The proteins in the Z line of insect flight muscle. *Biochem. J.* **161**, 399-403.

Bullard, B., Sainsbury, G. and Miller, N. (1990). Digestion of proteins associated with the Z-disc by calpain. *J. Muscle Res. Cell Motil.* **11**, 271-279.

Cohen, C. and Holmes, K. C. (1963). X-ray diffraction evidence for alpha-helical coiled-coils in native muscle. *J. Mol. Biol.* **6**, 423-432.

Epstein, H. F., Miller, D. M., 3rd, Ortiz, I. and Berliner, G. C. (1985). Myosin and paramyosin are organized about a newly identified core structure. *J. Cell Biol.* **100**, 904-915.

Epstein, H. F., Lu, G. Y., Deitiker, P. R., Oritz, I. and Schmid, M. F. (1995). Preliminary three-dimensional model for nematode thick filament core. *J. Struct. Biol.* **115**, 163-174.

Ferguson, C., Lakey, A., Hutchings, A., Butcher, G. W., Leonard, K. R. and Bullard, B. (1994). Cytoskeletal proteins of insect muscle: location of zeelins in *Lethocerus* flight and leg muscle. *J. Cell Sci.* **107**, 1115-1129.

Fürst, D. O., Osborn, M., Nave, R. and Weber, K. (1988). The organization of titin filaments in the half-sarcomere revealed by monoclonal antibodies in immunoelectron microscopy: a map of ten nonrepetitive epitopes starting at the Z line extends close to the M line. *J. Cell Biol*. **106**, 1563-1572.

Fyrberg, E. A. (1985). Genetic and Molecular Analyses of *Drosophila* Contractile Protein Genes. *Bioessays* **2**, 250-254.

Gregorio, C. C., Granzier, H., Sorimachi, H. and Labeit, S. (1999). Muscle assembly: a titanic achievement? *Curr. Opin. Cell Biol.* **11**, 18-25.

Henkin, J. A., Maughan, D. W. and Vigoreaux, J. O. (2004). Mutations that affect flightin expression in *Drosophila* alter the viscoelastic properties of flight muscle fibers. *Am. J. Physiol. Cell Physiol.* **286**, C65-72.

Karlik, C. C., Mahaffey, J. W., Coutu, M. D. and Fyrberg, E. A. (1984). Organization of contractile protein genes within the 88F subdivision of the *D. melanogaster* third chromosome. *Cell* **37**, 469-481.

Lakey, A., Ferguson, C., Labeit, S., Reedy, M., Larkins, A., Butcher, G., Leonard, K. and Bullard, B. (1990). Identification and localization of high molecular weight proteins in insect flight and leg muscle. *EMBO J.* **9**, 3459-3467.

Leonard, K. R. and Bullard, B. (2004). The thin filament in insect flight muscle. In *Nature's Versatile Engine: Insect Flight Muscle Inside and Out* (ed. J. Vigoreaux). Landes Bioscience: Georgetown, TX (http://www.eurekah.com).

Liu, F., Bauer, C. C., Ortiz, I., Cook, R. G., Schmid, M. F. and Epstein, H. F. (1998). β-Filagenin, a newly identified protein coassembling with myosin and paramyosin in *Caenorhabditis elegans*. *J. Cell Biol*. **140**, 347-353.

Liu, F., Ortiz, I., Hutagalung, A., Bauer, C. C., Cook, R. G. and Epstein, H. F. (2000). Differential assembly of α- and γ-filagenins into thick filaments in *Caenorhabditis elegans*. *J. Cell Sci.* **113**, 4001-4012.

Maroto, M., Arredondo, J. J., San Roman, M., Marco, R. and Cervera, M. (1995). Analysis of the paramyosin/miniparamyosin gene. Miniparamyosin is an independently transcribed, distinct paramyosin isoform, widely distributed in invertebrates. *J. Biol. Chem.* **270**, 4375-4382.

Maroto, M., Arredondo, J., Goulding, D., Marco, R., Bullard, B. and Cervera, M. (1996). *Drosophila* paramyosin/miniparamyosin gene products show a large diversity in quantity, localization, and isoform pattern: a possible role in muscle maturation and function. *J. Cell Biol.* **134**, 81-92.

Newman, R., Butcher, G. W., Bullard, B. and Leonard, K. R. (1992). A method for determining the periodicity of a troponin component in isolated insect flight muscle thin filaments by gold/Fab labelling. *J. Cell Sci*. **101**, 503-508.

Qiu, F., Lakey, A., Agianian, B., Hutchings, A., Butcher, G. W., Labeit, S., Leonard, K. and Bullard, B. (2003). Troponin C in different insect muscle types: identification of two isoforms in *Lethocerus*, *Drosophila* and *Anopheles* that are specific to asynchronous flight muscle in the adult insect. *Biochem. J.* **371**, 811-821.

Reedy, M. C., Bullard, B. and Vigoreaux, J. O. (2000). Flightin is essential for thick filament assembly and sarcomere stability in *Drosophila* flight muscles. *J. Cell Biol.* **151**, 1483-1500.

Sainsbury, G. M. and Bullard, B. (1980). New proline-rich proteins in isolated insect Z-discs. *Biochem. J.* **191**, 333-339.

Spellman, P. T. and Rubin, G. M. (2002). Evidence for large domains of similarly expressed genes in the *Drosophila* genome. *J. Biol*. **1**, 5.1-5.8 (doi:10.1186/1475-4924-1-5).

Szent-Györgyi, A. G., Cohen, C. and Kendrick-Jones, J. (1971). Paramyosin and the filaments of molluscan "catch" muscles. II. Native filaments: isolation and characterization. *J. Mol. Biol.* **56**, 239-258.

Trinick, J. (1996). Titin as a scaffold and spring. *Curr. Biol.* **6**, 258-260.

Vigoreaux, J. O. and Perry, L. M. (1994). Multiple isoelectric variants of flightin in *Drosophila* stretch-activated muscles are generated by temporally regulated phosphorylations. *J. Muscle Res. Cell Motil.* **15**, 607-616.

Vigoreaux, J. O., Saide, J. D., Valgeirsdottir, K. and Pardue, M. L. (1993). Flightin, a novel myofibrillar protein of *Drosophila* stretch-activated muscles. *J. Cell Biol.* **121**, 587-598.

Vigoreaux, J. O., Hernandez, C., Moore, J., Ayer, G. and Maughan, D. (1998). A genetic deficiency that spans the flightin gene of *Drosophila melanogaster* affects the ultrastructure and function of the flight muscles. *J. Exp. Biol.* **201**, 2033-2044.

Wray, J. S. (1979a). Filament geometry and the activation of insect flight muscle. *Nature* **280**, 325-326.

Wray, J. S. (1979b). Structure of the backbone in myosin filaments of muscle. *Nature* **277**, 37-40.

Zhang, S. and Bernstein, S. I. (2001). Spatially and temporally regulated expression of myosin heavy chain alternative exons during *Drosophila* embryogenesis. *Mech. Dev.* **101**, 35-45.